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Recent evidence suggests that EGF may influence human breast cancer progression via migratory pathways that, at least in part, appear to be dissociated from the proliferative pathways. We have shown previously that activation of the EGF receptor (EGFR) leads to tyrosine phosphorylation of the β4 subunit of α6β4 integrin, followed by disruption of hemidesmosomes and promotion of cell migration. The results reported here indicate that a fraction of EGFR is constitutively associated with α6β4. We also found that the tyrosine kinase Fyn mediates EGFR induced phosphorylation of the β4 cytoplasmic tail and disruption of the hemidesmosomes. Inhibition of Fyn activity leads to stabilization of hemidesmosomes and suppresses migration and invasion by breast carcinoma cells. These findings suggest that EGFR-mediated disruption of hemidesmosomes is a prerequisite for normal cell migration and tumor invasion.

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Introduction

Normal cell migration and tumor invasion both depend on changes in cytoskeletal organization and integrin function (1). Contraction of actin filaments and release of integrin attachments are both required for cell movement. Whereas β1 and αv integrins are connected to the actin cytoskeleton (2) and their role in migration and invasion is well characterized (3), the integrin α6β4 is associated with the keratin filaments (4, 5) and its role in the pathogenesis of invasive carcinoma is poorly understood. The integrin α6β4, a receptor for the laminins, is a component of hemidesmosomes (HDs), stable adhesion structures that anchor the basal layer of stratified epithelia to the underlying basement membrane (6). A number of modifications have been reported in α6 and β4 distribution pattern in most common histotypes of mammary tumors (7). Modifications include downregulation of the integrin or its redistribution over the plasma membrane of tumor cells. Other studies have indicated that the expression of the α6β4 integrin is associated with poor breast cancer prognosis, mainly in the laminin-producing tumor subset, suggesting that the interaction between α6β4 and laminin mediates signals that promote cell proliferation and/or invasion (8). Moreover, high levels of α6β4 integrin have been shown in many tumor cells that do not form stable adhesive contacts with the basement membrane, but rather exhibit the typical migratory ability of invasive carcinoma (9, 10). These findings suggest that the role of the integrin α6β4 in invasive tumors may be distinct from its well established function of anchoring the epithelial cells to the basement membrane. The understanding of the signaling pathway activated by this integrin during tumor progression should provide important insight into the mechanism of invasion.

It has been recently reported that EGF may influence human breast cancer progression via migratory pathways that, at least in part, appear to be dissociated from the proliferative pathways (11). Previous studies from our lab have shown that activation of the EGF receptor (EGFR) leads to tyrosine phosphorylation of the β4 subunit,

followed by disruption of HDs and promotion of cell migration (12). We have investigated in more detail the molecular mechanisms by which EGF stimulation affects α6β4 cellular functions and how the uncontrolled cross-talk between the two receptors can influence the progression of breast carcinoma. The results reported here indicate that a fraction of EGFR is constitutively associated with α6β4. We also found that EGFR induces the activation of the tyrosine kinase Fyn, and this activation is required for the phosphorylation of the β4 cytoplasmic tail and disruption of the HDs. Inhibition of Fyn activity leads to stabilization of the HDs and suppresses migration and invasion by breast carcinoma cells. These findings suggest that EGFR-mediated disruption of HDs is a prerequisite for normal cell migration and tumor invasion.

Body

Previous studies had shown that exposure to physiological concentrations of EGF induces phosphorylation of multiple tyrosine residues within the cytoplasmic domain of β4 in a variety of normal and transformed cells (12). To investigate in more detail the activation of β4 by EGFR and the biological significance of this event in breast cancer progression, experiments of co-immunoprecipitation, followed by immunoblotting with specific antibodies, were performed. Total extracts were prepared from unstimulated as well as EGF-stimulated breast carcinoma cells, immunoprecipitated with a monoclonal anti-β4 antibody (3E1) and subjected to SDS/PAGE, followed by immunoblotting using specific antibodies (Fig. 1). Immunoblot with an anti-EGFR antibody showed that a significant fraction of the EGFR is constitutevely bound to α6β4. This association is specific, since no association of β4 with Erb-2, another member of the EGFR family, was detected. Immunoblot with anti-phosphotyrosine (anti-P-Tyr) antibody showed that, upon EGF stimulation, β4 as well as the β4-associated EGFR fraction become phosphorylated. Moreover, western blot analysis with anti-EGFR antibody showed that no further association of EGFR with β4 is induced by EGF treatment.

Since previous studies had shown that the EGFR does not phosphorylate β4 directly in vitro (12), we decided to identify the tyrosine kinase responsible for EGF-induced phosphorylation of β4. By using increasing amounts of the newly described src-family kinase specific inhibitors PP1 and PP2 (13), we observed a decrease in β4 tyrosine phosphorylation in response to EGF stimulation (Fig. 2A, B). The treatment with the inhibitors does not affect EGFR activation. These results suggested that a src-family kinase is activated by EGF and is, at least in part, responsible for subsequent β4 phosphorylation. In order to identify the src-kinase responsible for β4 activation, cotransfection experiments were performed. A plasmid expressing α6β4 was transiently introduced, together with single src-family kinases, in 293T cells. Cell lysates were immunoprecipitated with specific antibodies anti-β4, or anti-fyn or anti-src and immuno-complexes subjected to an in vitro kinase assay. Results in Fig. 3 show that the tyrosine kinase fyn strongly binds to α6β4, while no association of src tyrosine kinase is found, suggesting that fyn may be the tyrosine kinase that phosphorylates β4 in response to EGF. In order to verify this assumption, we stably transfected the squamous carcinoma cell line 804G, which forms HDs in vitro, with vectors expressing dominant negative fyn or src kinases (DNfyn, DNsrc). Several clones expressing DNfyn (F1, F2, F3) or DNsrc (S1, S2) were isolated (Fig. 4A). We observed that tyrosine phosphorylation of β4 after EGF treatment is decreased in clones expressing DNfyn but not in control or in DNsrc-expressing clones (Fig. 4B). In addition, immunofluorescence staining with 3E1 antibody revealed an increased number of HDs in DNfyn-expressing clones, compared to the control and DNsrc-expressing clones. Moreover, in the presence of DNfyn, HDs become more stable and are only partially disassembled after EGF stimulation. Taken together, these results suggested that fyn tyrosine kinase is the signaling molecule mediating the cross-talk between EGFR and β4. It is responsible for β4 activation upon EGF stimulation and it is specifically involved in the HDs turnover induced by the growth factor. These functions of fyn on β4 integrin can not be substituted by any other src-family kinase.

We then examined whether the increase in HDs number and stability promoted by DNfyn could affect cellular responses stimulated by EGF, such as cell migration and invasion. A wound assay on the 804G clones revealed that DNfyn reduced EGF-stimulated cell migration to a greater extent than DNsrc. An invasion assay, through a reconstituted basement membrane (Matrigel), showed that either DNfyn or DNsrc strongly inhibited cell invasion induced by EGF. In order to verify the role of src-family kinases in cell invasion, breast carcinoma cells were tested for their ability to migrate through Matrigel, in response to EGF, in the presence of the src-family inhibitor PP1. We found that EGF-induced invasion ability of breast carcinoma cells was strongly inhibited by PP1, confirming a role for the src kinases in tumor invasion.

Discussion:

Taken together, our results demonstrate that the src-family member fyn mediates signaling between the EGF receptor and the laminin receptor $\alpha 6\beta 4$. We demonstrated that Fyn induces activation of $\beta 4$ upon EGF stimulation and HD disruption, which is a prerequisite for tumor progression. We also show that both fyn and src kinase activities appear to be required for breast carcinoma invasion stimulated by EGF. These findings suggest a mechanism by which deregulated cross-talk between the two receptors can promote breast carcinoma progression.

Key Research Accomplishments

- EGF receptor associates with the integrin α6ß4
- Fyn tyrosine kinase is activated by EGF receptor
- Fyn kinase activity is required for phosphorylation of ß4 in response to EGF receptor activation
- Fyn kinase activity is required for hemidesmosome disruption after EGF stimulation
- Disruption of hemidesmosomes by Fyn activity is a prerequisite for EGF stimulated cell migration and invasion

Reportable Outcomes

Development of squamous carcinoma 804G cells expressing dominant-negative Fyn or dominant-negative Src

Conclusions

Our results demonstrate a role for the src-family member fyn in the signaling between the EGF receptor and the laminin receptor α6β4. EGF stimulation induces activation of Fyn, which in turn phosphorylates β4 causing disruption of hemidesmosomes. As disruption of hemidesmosomes is a prerequisite for tumor progression, these results suggest that suppression of Fyn activation may inhibit tumor invasion. In agreement with this, we also find that both fyn and src kinase activities are required for breast carcinoma invasion stimulated by EGF. Our findings suggest a mechanism by which deregulated cross-talk between the two receptors can promote breast carcinoma progression and suggest that development of drugs that suppress this signaling may provide novel tumor therapies.

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Appendices

4 figures

Final Report: At present there have been no publications or abstracts as a result of this research. No personnel received pay from these funds.

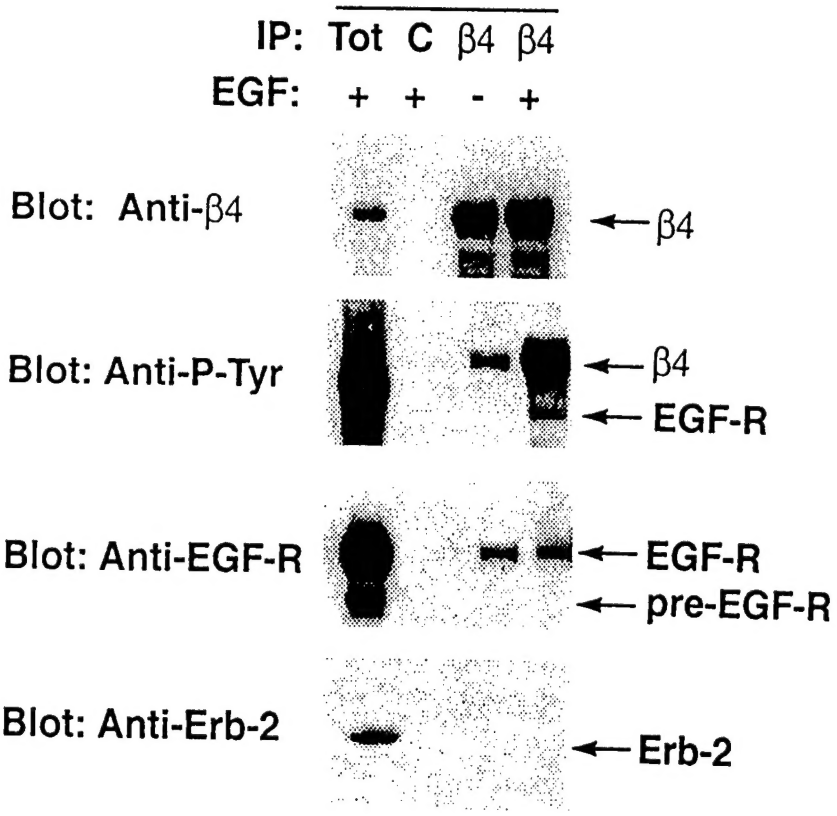

Fig. 1 Association of EGFR with the integrin α6β4 and activation of α6β4 after EGF treatment. Lane 1: total extract; lane 2: IP with a non specific antibody; lanes 3 and 4: IP with the 3E1 monoclonal antibody . The association between EGFR and α6β4 was determined by immunoblotting with anti EGFR antibody. The activation of β4 was determined by immunoblotting with an anti-phosphotyrosine antibody.

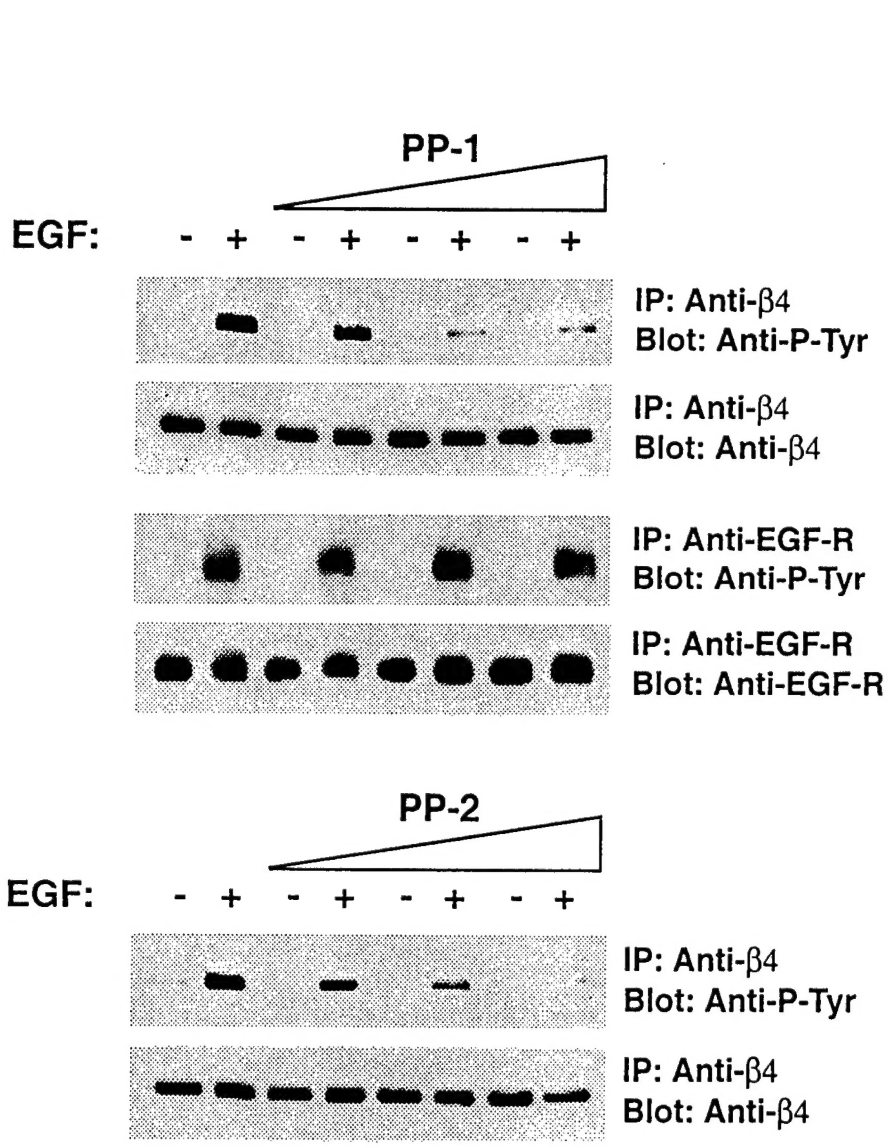

Fig. 2 Effects of PP1 and PP2 src-family kinase inhibitors on β4 phosphorylation upon EGF stimulation. Cells were stimulated with EGF in presence or absence of increasing concentration (0.1, 1.5, and 10μM) of PP1 (panel A) or PP2 (panel B) inhibitors and phosphorylation of β4 and EGFR determined by immunoblotting with anti-P-Tyr antibody. Blots with anti -β4 and anti-EGFR antibodies were performed as loading control.

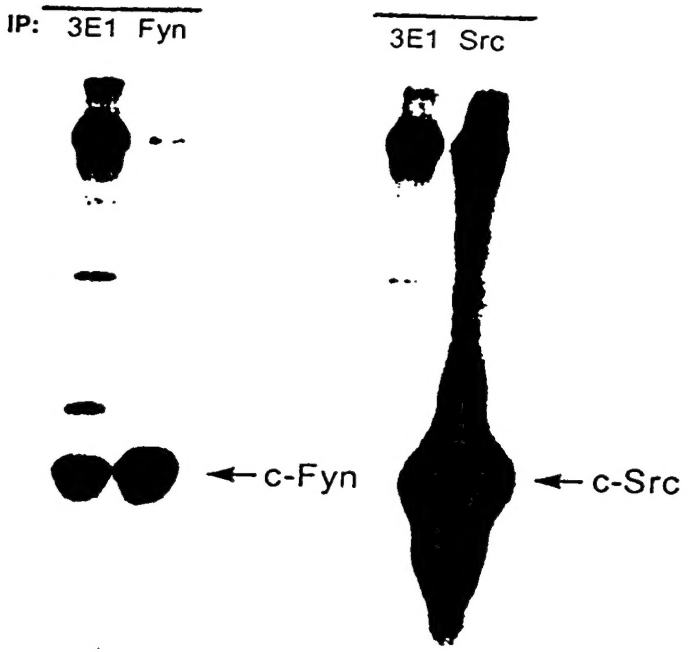

Fig. 3 In vitro kinase assay showing the association of Fyn with $\alpha 6\beta 4$ in transfected 293T cells. Transiently transfected cells were immunoprecipitated with the 3E1 antibody or an anti-Fyn antibody or an anti-src antibody. The immuno-complexes were incubated with ^{32}P-γATP, before SDS-PAGE analysis.

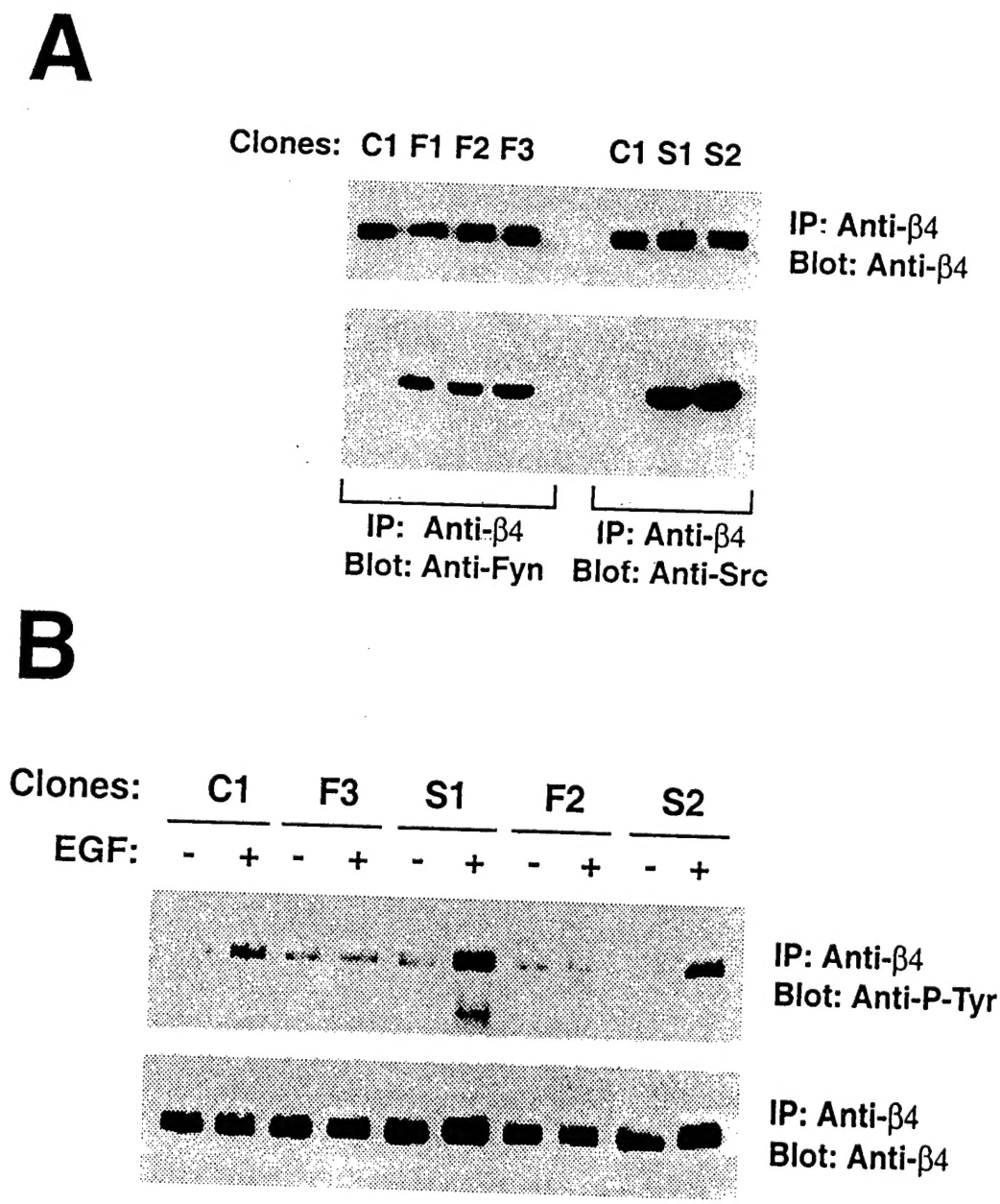

Fig. 4 Panel A: 804G clones expressing DNfyn and DNsrc. F1, F2, F3 are clones expressing DNfyn. S1 and S2 are clones expressing DNsrc. C1 is the parental cell line transfected with the vector alone. Expression of DNfyn or DNsrc in the different clones was determined by immunoblot. Panel B: Phosphorylation of β4 upon EGF stimulation in DNfyn and DNsrc clones. Tyrosine phosphorylation of β4 is significantly reduced in DNfyn expressing clones.